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Characterisation of the type and extent of nutrient limitation in grassland vegetation using a bioassay with intact sods

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Abstract

An important methodological problem in plant ecology concerns the way in which the type and extent of nutrient limitation in terrestrial communities should be assessed. Conclusions on nutrient limitation have been founded mainly on soil extractions, fertiliser trials and tissue nutrient concentrations. In order to avoid some of the problems associated with these methods, we employed a special technique using intact sods which rooted both in the intact soil and in a nutrient solution, from which N, P and K were omitted stepwise. The method was applied to hay-field communities which differed in their history of fertiliser application. Four fields were compared which were not fertilised for 2, 6, 19 or 45 years, while hay making continued. This was done to restore former species-rich grassland communities. We tested the hypothesis that the increase in species diversity in these grasslands was attended by an increase in the number of limiting nutrients.

We observed clear shifts in the type and extent of nutrient limitation. Fields which were recently fertilised were characterised by nitrogen and potassium limitation, while phosphorus limitation increased in importance towards the later stages of succession. In the last field (45 years unfertilised) N, P and K equally limited production at the community level. These conclusions differed from those drawn from a fertiliser trial in these same four fields, which failed to detect phosphorus limitation.

It is concluded that the use of this method provides a valuable extra source of information while studying relationships between nutrient limitation and species diversity in grassland communities.

Introduction

The problem of nutrient limitation in plants was first thoroughly studied by Von Liebig (1840), resulting in his 'Law of the Minimum'. He concluded that individual plants can be characterised by a fixed order of limitation of each of their nutrients, of which only one actually limits growth. This implies that the difference between actual growth and growth with all nutrients in excess of demand (but non-toxic), can be used as an index of nutrient limitation (Chapin et al., 1986). However, plants are not fully helpless against a change in the supply rate of a specific resource in their environment. Since various plant organs and functions (as chloroplasts, stomata, root hairs) are each more or less specialised in the acquisition of certain (groups of) resources, plants can allocate more effort (energy, carbon, nutrients) to uptake of a limiting resource to avoid potential overall resource imbalances (Bloom et al., 1985; Bradshaw, 1965; Brouwer 1963). However, plasticity of allocation patterns offers little possibility of coping with imbalances between nutrients, since investment of biomass in more roots usually leads to an increased uptake of all nutrients simultaneously (Gleeson and Tilman 1992). A species can only avoid nutrient imbalances by reducing its demand (low tissue concentrations, low growth rate), by reducing its losses of that nutrient (Chapin, 1980; Shaver and Melillo 1984; Small, 1972) or by specific uptake mechanisms (Fitter and Hay, 1981).

A set of low supply rates accompanied by soil heterogeneity and species-specific differences in uptake, requirements and losses may be one of the explanations why many species can coexist in unproductive communities (Tilman, 1982). The type and extent of nutrient limitation can be determined for individual species, as well as at the level of total productivity of the community.

The degree of nutrient limitation of an individual plant, of a species, or of the whole community will be jointly determined by the balance between the supply of nutrients and by the demand. Inherently fast growing species (high demand) will become limited when the supply is only slightly reduced. At such a level of supply, it may still exceed the demand of inherently slow growing species. Several methods have been used to investigate supply, demand, and their balance. Soil analysis of supply may involve repeated extractions (e.g. Troelstra et al., 1990) and measurement of mineralization rates (e.g. Raison et al., 1987). Analysis of plant tissue nutrient concentrations have been performed to determine critical levels below which the plant is assumed to be limited by that nutrient (Bates, 1971; Van den Driessche, 1974; Wentworth and Davidson, 1987). The balance between supply and demand has been investigated in fertiliser application experiments (DiTomasso and Aarssen,1989) and in phytometer tests (e.g., Pegtel, 1983, 1987; Van der Woude et al., 1994).

However, each method has certain drawbacks. Point extractions of the available form of a nutrient may show results that are very variable in time, and are often poorly related to fluxes. Furthermore, different extractants may provide different results, especially for phosphorus, which makes it difficult to decide which extraction reflects the pool size which is available to a species (Williams and Knight, 1963). Another problem is that most extractions are developed for agricultural conditions, and therefore fail to discriminate between low levels which may exist in natural communities. An additional problem with extraction methods is that plants differ in their ability to modify the root environment, the effect of which is difficult to mimic with an extraction. Mineralization measurements (field or laboratory incubations) seem to work for nitrogen in many cases. For phosphorus however, mineralization studies can only be applied in purely organic soils. Nutrient additions in fertiliser trials may be strongly immobilised by inorganic adsorption processes (fixation) and chemical solubility phenomena (chelation).

Especially for phosphorus these phenomena seem to be quite important (Chapman et al., 1989; Sharpley et al., 1984; Van der Woude et al., 1994). Nitrogen added to wet soils may be lost by denitrification. Summarizing, this implies that a low response to nutrient addition of a natural plant community does not necessarily mean that productivity is not limited by that nutrient (Chapin et al., 1986). A high response of total productivity on the contrary usually means that only a few fast growing species are monopolising the added nutrients (see e.g., Tilman, 1987; Willis, 1963). Tissue nutrient concentrations do not only reflect differences between habitats, but also will interact strongly with the species used. The same problem holds for phytometer experiments, where different test species will yield different results.

The present study was set up to exploit the potentials of a method which avoids some of these difficulties. We tested this method by comparing four contrasting grassland communities which differed in species diversity, which allowed us to explore the relationships between the number of limiting nutrients and the number of coexisting species.

Materials and methods

Outline of the method

Intact soil cores with the original grassland vegetation were placed on a perforated plate above a nutrient solution in a greenhouse (Fig. 1). After a few weeks, the vegetation started to root both in the soil and in the nutrient solution, from which specific nutrients were omitted stepwise. When a single nutrient is omitted from the solution in this set-up, then the soil supply is the only source of that nutrient for the plant community. By adding the other nutrients in excess to the solution, a strong demand is created for the nutrient which is omitted. The maximal demand (potential productivity of that particular community) is obtained when all nutrients are added to the solution. The relative reduction which follows after omitting a single nutrient is then a good reflection of the supply rate of that nutrient by the soil, and is made independent from the absolute demand. When the yield is greatly reduced, this means that the soil of that particular community has a low supply, relative to the potential demand by that community. This means that differences between communities (caused by differences in species composition) in ability to respond to extra nutrients are accounted

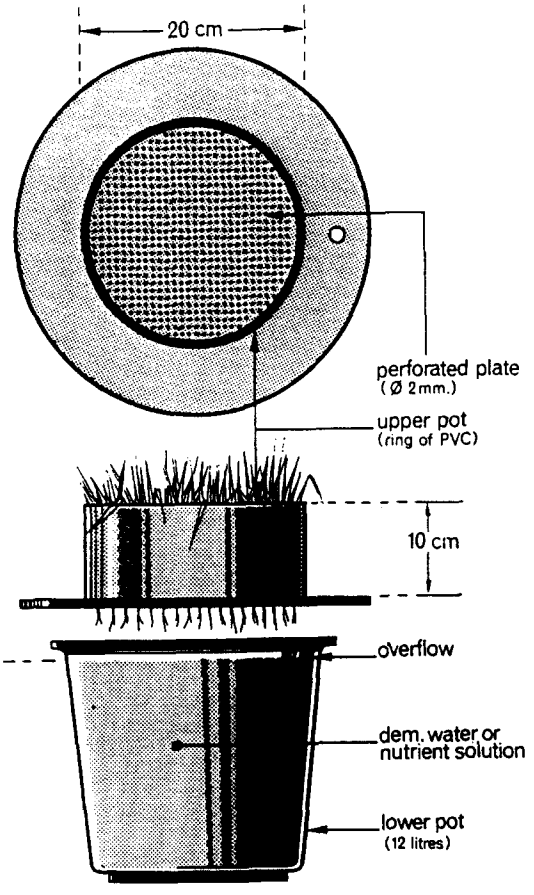

Fig. 1. Experimental set-up for the assessment of nutrient limitation in intact sods. The vegetation was rooting both in the soil and in various nutrient solutions, from which specific nutrients were omitted stepwise.

for. Further advantages of the method are that accumulation, adsorption, fixation or microbial transformations (e.g., denitrification) of added nutrients are avoided, since the nutrient solution and the soil are separated. Furthermore, by replenishing the solution the same concentrations of the available forms can be maintained.

Detailed experimental procedures

Undisturbed, circular sods (20 cm, 10 cm depth) were collected on 11 and 12 April 1991, using a specially designed soil corer containing a PVC-ring. The ringed sods were placed on perforated plates (with 2-mm holes) which were placed on top of 12L buckets. The buckets were initially filled with demineralised water. After one month, roots had grown into the buckets, and the demineralised water in most pots was replaced by various nutrient solutions, while one series kept the demineralised water as a control. The water table of the nutrient solutions was kept 1 cm below the perforated plates. The complete solution was based on an equivalent ratio of 35 K^+, 45 Ca^{2+}, 20 Mg^{2+}, and 60 NO_3^-, 5 $H_2PO_4^-$ and 35 SO_4^{2-}. The total ion concentration was 20 m*M* which corresponds theoretically with an osmotic potential of -0.48 bar at 20 °C. Micronutrients (in μmol L^{-1}) were: 35 Fe (as Fe-DTPA), 10 Mn (as $MnSO_4.H_2O$), 3 Zn (as $ZnSO_4.7H_2O$), 20 B (as $Na_2B_4O_7$. $10H_2O$), 0.5 Cu (as $CuSO_4.5H_2O$) and 0.5 Mo (as $(NH_4)_6Mo_7O_{24}$)

We compared the effects of demineralised water (control), a complete nutrient solution, and three nutrient solutions lacking the macronutrients N, P or K, respectively. Each treatment was replicated 4 times (using 4 sods from each field). This yielded 4 × 5 × 4 = 80 sods in total. Sodium was substituted for potassium (K-deficient solution), and chloride for nitrate or phosphate (N-deficient or P-deficient solution, respectively).

All sods were randomly placed in a heated greenhouse with a minimum temperature of 16 °C and long-day illumination (16 h) and were watered daily with demineralised water. The aboveground vegetation was clipped to 3 cm height on 29 May (46 days), 8 July (86 days), 16 August (125 days) and 24 September 1991 (164 days). The nutrient solutions were changed after each clipping. The clippings were hand-sorted to the following species groups: grasses (including sedges), dicots, rushes (Juncaceae) and legumes. The samples were dried at 70 °C and weighed.

Site description

The sods were taken from a chronosequence of four moist grassland fields, which had not been fertilised for 2 (Field A), 6 (Field B), 19 (Field C) or 45 years (Field D). These fields are cut for hay each year in July. Field A was dominated by *Agrostis stolonifera* and *Poa trivialis*, field B by *Holcus lanatus*, field C by *Anthoxanthum odoratum* and *Holcus lanatus*, and field D by *Juncus acutiflorus*. These four fields represented different stages of a successional series which started after the application of fertilisers to these grasslands was stopped but hay making was continued (Bakker, 1989; Olff and Bakker, 1991). Hay making is performed by the nature management authorities to restore former species-rich grassland communities which existed here until the 1920's, when artificial fertilisers were first applied. The progress of vegetation succession in field C has been studied for the last 20 years and it was found that the vegetation in this field after 2 and

6 years of succession closely resembled the vegetation now found in fields A and B, respectively (see Bakker, 1989; Bakker and Olff, 1994). The annual in situ N mineralization rate was found to be lowest in field D (Olff et al., 1994a). The vegetation composition in field D before 1946 (the period of its agricultural use) is not known; it is however likely that the agricultural use in this field before the start of the nature management practices was less intensive than in the other three fields. The fields are located along a small brooklet in a glacial sand plain area (Anloërdiepje, 53°05' N, 6°40' E). Each field contains a topographic gradient ranging from the sandy plateau to the brooklet. The sods were sampled in the lower, wetter part of this gradient.

Data analysis

A statistical comparison of the overall differences between fields, treatments and harvest times of ln-transformed total yield per pot was done in a three-way analysis of variance, with Student-Newman-Keuls contrasts among means. Homogeneity of variances was checked using Cochran's C test. The nature of interactions between treatments and fields (differences in nutrient limitation between fields) were investigated further by calculation of the cumulative yield per pot over the four harvests, and comparing differences in this measure between treatments within each field. Differences between fields in mean cumulative yield at full nutrient solution were very small, indicating that is was not necessary to take these differences into account (e.g. by standardising all treatments within a field towards this total). By comparing the effects of omitting each nutrient on cumulative yield (compared with full nutrient solution) we gained insight in which nutrients were limiting productivity in each field. Differences between treatments within a field and harvest time were tested using Student-Newman-Keuls contrasts among means after one-way analysis of variance.

Results

The total dry matter yield per pot for each combination of field, treatment and harvest time is presented in Figure 2. Three-way analysis of variance for testing the effects of field, treatment and harvest on ln-transformed total yield per pot revealed that all main effects and two-way interactions were significant ($p < 0.001$). Each main effect was investigated further by Student-Newman-Keuls contrasts among means, to test for overall differences (pooled over the other factors). This revealed for the field effect ($F_{3,240} = 26.4$ $p < 0.001$) that, averaged over all treatments and harvests, field A and field B both had a higher yield than field C and D, while within these pairs, fields were not significantly different. Further investigation of the harvest effect ($F_{3,240} = 26.4$ $p < 0.001$) showed that the second and third harvest had a significantly higher yield than the fourth harvest, all other comparisons did not provide significant differences ($p > 0.05$). The overall means per treatment were ranked from low to high as: control, minus K, minus N, minus P and full nutrients. Contrasts for this treatment effect ($F_{4,240}$) revealed that all pair wise comparisons were significant ($p < .05$), except for the difference between minus K and minus N.

Despite these general differences between fields and treatments, these differences were not fully consistent between harvests. At the first harvest, differences between treatments were relatively small. The effect of omitting N was large at harvest 2 and 3, but lower at harvest 4. Effects of omitting K and P were consistent between harvests 2, 3, and 4.

The results of the cumulative analysis (summed over all harvests) are given in Figure 3 . The cumulative yield per treatment per field is subdivided for the four species groups, but differences were tested using the summed yield of these four groups. The effect of omitting all nutrients simultaneously resulted in the largest yield reduction in all fields. However, substantial differences were observed in the effect of the other treatments. Field A and B were limited by both N and K, an effect which also occurred in fields C and D. The effect of omitting K was larger in fields C and D than in the earlier successional fields. Furthermore, omitting P in field C significantly reduced the cumulative yield towards the full nutrients treatment. The magnitude of this effect was larger still in field D. This means that during succession nutrient limitation changed from both N and K during early succession (recent cessation of fertiliser application), to simultaneous N, K and P limitation in the later fields of the chronosequence. In field D, the effect of omitting either N, P, or K was almost as large as omitting N, P and K simultaneously (Fig. 3).

There seemed to be some treatment-dependent differences between the various species groups in their contribution to the cumulative yield (Fig. 3). The contribution of non-leguminous forbs was larger in the full nutrients treatment than in the other treatments, in every field. The contribution of rushes in field D

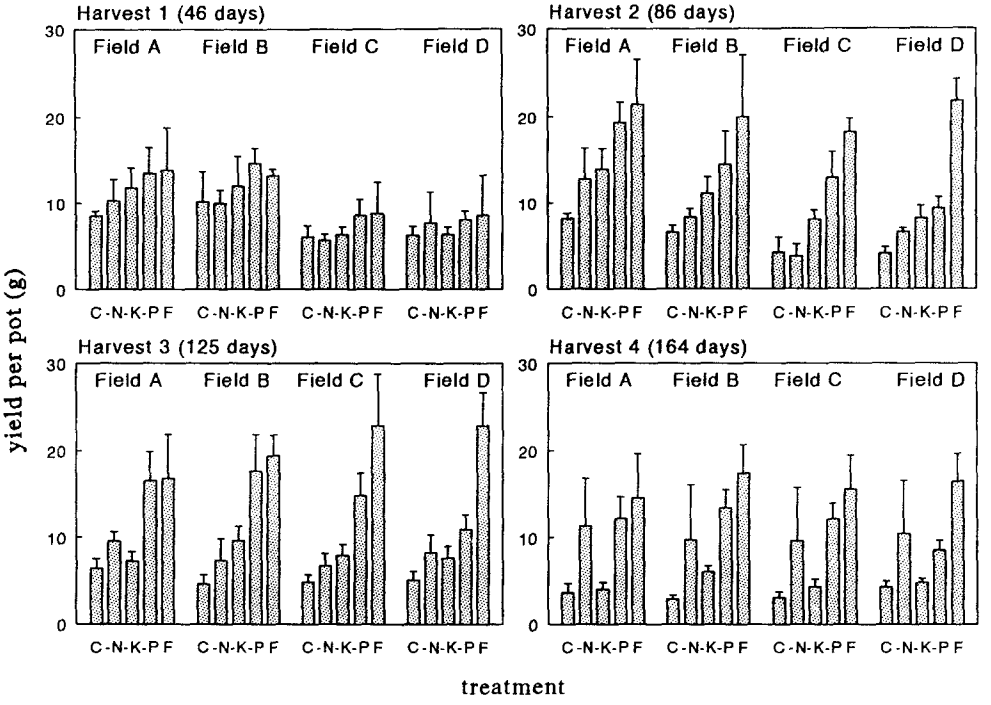

Fig. 2. The effect of omitting various nutrients from a nutrient solution (see Fig. 1) on the shoot dry matter yield per pot of undisturbed sods, originating from four successional hayfields which were not fertilised for various periods of time (Field A — 2 years, Field B — 6 years, Field C — 19 years, field D — 45 years), when grown with the roots in nutrient solutions which was either complete (F), N deficient (-N), K- deficient (-K), P-deficient (-P) or lacking N, P and K (C). Harvest times were: (1) 46 days, (2) 86 days, (3) 125 days and (4) 164 days. Average values (n = 4) with standard deviation are given.

was larger in the minus N and the minus P treatment. At full nutrient solution, the rushes did not increase in comparison with the control, the increased yield in this treatment was fully accounted for by grasses and non-leguminous forbs. Legumes increased in field D in the minus N treatment compared with the control, and to a lesser extent in the minus P treatment. In the other fields, legumes and rushes were not important.

Discussion

Usefulness of the method

The technique employed in our experiment for the assessment of the type and extent of nutrient limitations has several advantages over more widely-used methods: (i) it ensures that the sod response measured is (in the short run) ecologically real, because the response of plant species in their original setting is assessed, and (ii) inorganic adsorption/fixation processes and immobilisation by micro-organisms, as might occur in field fertiliser application trials, are avoided. In soils which are affected by Fe-rich seepage groundwater- as these soils are (Pegtel, 1987) — precipitation of inorganic phosphate can be an important process, resulting in very low levels of plant-available P (Boyer and Wheeler, 1989; Van der Woude et al., 1994).

Besides these advantages, our method also has some unavoidable shortcomings. The method implicitly assumes that the component plant species all have the same ability to develop new roots through the perforated plate into the nutrient solution or water. We do not have information yet on the validity of this assumption. Furthermore, changes in plant species composition during the experimental period may influence the plant community response. The variation through time then also becomes a function of changing demand. The frequent clipping during the experiment is needed to maintain growth during a longer period: specific nutrient deficiencies may become more apparent and self-shading of the canopy is restricted. However, it favours those plant species which are the most tolerant to clipping, possibly contributing to species replacement. We applied the method to grassland communities which are cut every year, and the component species

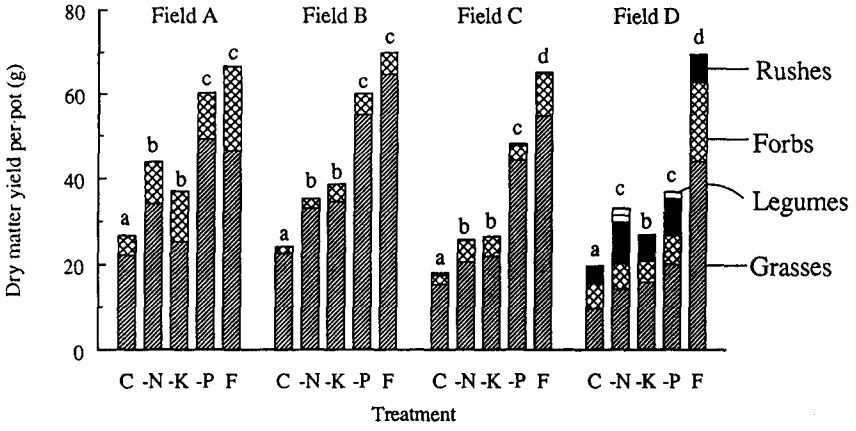

Fig. 3. The effect of omitting various nutrients from a nutrient solution (see Fig. 1) on the cumulative shoot dry matter yield (over four harvests) per pot (n = 4) of undisturbed sods, originating from four successional hayfields which were not fertilised for various periods of time (Field A — 2 years, Field B — 6 years, Field C — 19 years, field D — 45 years), when grown with the roots in nutrient solutions which were either complete (F), N deficient (-N), K-deficient (-K), P-deficient (- P) or lacking N, P and K (C). Bars with the same letter were not significantly different ($p > 0.05$), within each field (Student-Newman-Keuls contrasts after one-way analysis of variance).

are therefore expected to be rather tolerant to clipping. If, however, the method is applied to plant communities not subjected to cutting and/or grazing, than a single end-harvest would be more appropriate. However, our results showed that some time was needed in order for the effects to emerge. During the first harvest, little differences between treatments were observed. Although the sods had some roots in the solution at this moment, they probably did not yet make a large contribution to the overall nutrient supply. At the fourth harvest, the effect of omitting N decreased again. This could perhaps be due to mineralization of N in the sods from dying roots (dying due to frequent clipping). For an 'ecologically real' result therefore, the experiment should not last too short or too long (4–5 months).

Despite these aforementioned disadvantages, the methods provided several important insights into the nature of nutrient limitation of this successional series, especially when combined with information from other experiments, such as effects of fertiliser application in the field and tissue nutrient concentrations of several species.

Nutrient limitation, yield and species diversity

The sods from the two relatively nutrient-richest successional stages (just recently fertilised) had a higher shoot dry-matter yield than the two relatively nutrient-poorer stages (fields C and D). This difference could mainly be attributed to a higher yield of these two fields early in the experiment. Possible explanations for this could be a higher initial nutrient availability in the intact soil in these fields, or a higher overall growth rate of the component plant species of these fields. Since the differences were initially present at all treatments and decreased during the experiment, the first explanation is more likely.

The control treatment (no nutrients added) caused the largest reduction of total shoot yield, followed by equally effective N- and K- deficiency. The extent of omitting potassium in this experiment was striking, and this effect increased during the chronosequence. The overall degree of nutrient limitation (control compared to full nutrient solution) was the largest in the two fields which had been subjected to the relatively longest periods of hay making without the application of fertilisers (fields C and D). In a complementary study, it was found that the in situ N-mineralization in these two fields during the summer period was much lower than in fields A and B, attended by lower nitrification rates (Olff, 1994a).

It was found that N- and K-limitation were especially important in the fields where fertiliser application stopped just recently, while P-limitation became gradually more important in the fields which were not fertilised for a long time. A first possible explanation for this difference in the rate at which these nutrients become limiting, is the different behaviour of these nutrients in the soil. Nitrate, ammonium and potassium are highly mobile in the soil, while phosphate is less mobile due to adsorption phenomena. Since biogeochemical recycling and weathering of potassium

is usually low, fertiliser application must have been main source of K in these grasslands. Given that these soils are low in clay content (Olff, 1992a) from which weathering could occur and K is highly mobile, the applied K is expected to have been leached quickly. This may explain the increase in K limitation towards the older stages of succession. Therefore the disappearance from the system of fertiliser N and K due to leaching is probably quicker than for P. However, the observed changes in the type of nutrient limitation with decreasing total supply rate might also be related to nutrient-specific patterns of storage and distribution within the plant. Plant species characteristic of man-managed grasslands with a relatively high total nutrient supply rate are usually physiologically characterised by a high investment of N in proteins involved in photosynthesis (chlorophyll and RUBISCO), a high growth rate and little storage in roots. A high growth rate can be important under productive conditions with yearly losses of most above-ground biomass, where the fastest growing and tallest species get the light first (Olff, 1992b). High N-concentrations in the leaves are, however, also usually attended by high turnover rates of the leaves which consequently increases the demand of N by the plant (Berendse et al., 1992; Shaver and Melillo, 1984). Combined with the high proportional losses of N (and probably also K) when the sward is cut each year, species with such high losses of N can only be dominant under high supply rates of N (Berendse et al., 1992; Shaver and Melillo, 1984). Analysis of internal nutrient concentrations of the dominant plant species in our four fields (Olff, 1992a) indeed showed that the concentrations of N and also K were higher in the shoots than in the roots, especially in the dominants of the earlier successional stages. The distribution of P over shoots and roots was more similar. Therefore, cutting a productive grassland community causes a proportionally higher loss of N and K (as a fraction of the internal pool), as compared to cutting an unproductive grassland community. The proportional losses of P will be more alike both in productive and unproductive grasslands. Since lost nutrients have to be compensated for by increased uptake in order to restore the previous level of productivity, productive communities have a high demand for N and K, and can therefore be limited relatively rapidly if the supply rates of these nutrients by the soil is not adequate for such types of plant species. P- limitation then only becomes important in unproductive grasslands. This hypothesis however has to be investigated further. The increasing importance of P limitation appeared to be a gradual process where the extent limitation increased until all nutrients became finally limiting. An interesting comparison can be made between this results and those of a factorial fertiliser application experiment in these same four fields (Olff, 1992b). Application of 8 g P m^{-2} y^{-1} in field D over two years did not have any significant effect on root or shoot biomass. This might therefore be an example of a situation where added nutrients were precipitated in the soil, thereby failing to show that productivity was indeed limited by P. The results with respect to N and K limitation were the same for both methods.

The shift from a single nutrient limitation in the productive community to multiple nutrient limitation in the unproductive community is very interesting in relation to changes in plant species diversity in these grasslands. The generally found increase in plant species diversity with decreasing productivity (Grime, 1979) was also found during our studied successional sequence: it increased from about 5 to 40 species per 4 m^2 (Bakker, 1989; Olff and Bakker, 1991). Decreasing productivity attended by some soil heterogeneity may lead to increased possibilities for (i) colonisation and establishment by plant species arriving from elsewhere (Olff et al., 1994b), and (ii) subsequent coexistence, where each species is limited by a different resource (Tilman, 1982). Whenever resource competition is the main factor controlling species composition, the number of coexisting species in a homogeneous environment is expected to equal the number of limiting resources. With some heterogeneity in supply rates however (see Beckett and Webster, 1971), the number of coexisting species may be much larger (Tilman, 1982). It was indeed found that species diversity increased with the number of nutrients which was limiting in each community. It should however be noted that this observed correlation is not really a proof for any underlying mechanisms. This should be given by further experiments on the outcome of competition at different ratios of nutrient supply rates and by the analysis of patterns of heterogeneity in supply rates in the field.

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